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Cancer Immunol Immunother. 1995 Jun;40(6):367-75.

Cancer Immunol Immunother. 1996 Feb;42(2):81-7.

Clin Cancer Res. 1998 Aug;4(8):1921-9.

Int J Pancreatol. 1997 Feb;21(1):43-52.

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Immunopathology of Metastases in Patients of Colorectal Carcinoma Treated with Monoclonal Antibody 17-1A and Granulocyte Macrophage Colony-stimulating Factor¹

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ABSTRACT

Twenty patients with metastatic colorectal carcinoma were treated with a single infusion (400 mg) of a mouse monoclonal antibody (IgG2a) against the tumor-associated antigen CO 17-1A and with a daily injection of granulocyte macrophage colony-stimulating factor (GM-CSF) for 10 days. The cycle was repeated every month. Metastases from 5 of the 20 patients biopsied on days 1 and 10 of the first two treatment cycles were studied by immunohistochemistry.

During treatment, neutrophils, monocytes, and T lymphocytes increased concordantly in the tumor as in the blood of the individual patient. Macrophages (CD68) and CD8⁺ T cells infiltrated the tumor glands and displayed TIA-1-reactive cytotoxic granules. Neutrophils were seen mainly in areas of necrosis. Activated (HLA-DR⁺) CD4⁺ T cells were usually abundant in the stroma. During treatment, few natural killer cells were found in the tumor, contrary to the marked increase seen in blood. Our observations indicate that GM-CSF markedly recruited activated, tumor-infiltrating leukocytes, possibly representing antibody-dependent cellular cytotoxicity and cytotoxic T effector cells. The notion that combined antibody and GM-CSF therapy may also promote a T-cell antitumor response is further supported and advocated by our findings. The study lends further

support to combining GM-CSF with monoclonal antibody-based therapy.

INTRODUCTION

The poor prognosis of metastatic CRC³ treated with chemotherapy obviates the need for alternative treatment modalities. Previous studies with MAbs, alone or in combination with other biological agents, indicated antitumoral effects in human xenografts and CRC patients (1–3).

Unconjugated MAbs can destroy tumor cells by various direct mechanisms, such as ADCC (4), complement-dependent cytosis (5), and apoptosis (6), or indirectly by the induction of an idiotypic network response (antitumor immunity; Ref. 7). Patients treated with MAb developed anti-idiotype antibodies (ab_2), which bound to the variable region of the therapeutic MAb (ab_1 ; Ref. 8). According to the idiotypic network theory, within the ab_2 variable region, there is a mimicry of the epitope that ab_1 recognizes, i.e., the nominal antigen. Correspondingly, T cells (T_2) against the idioype of ab_1 may also be induced (9). Such an ab_2/T_2 mimicry can subsequently evoke an anti-anti-idiotype response, with the production of anti-anti-idiotype antibodies (ab_3) and T cells (T_3 ; Ref. 10), which may recognize the same epitope as ab_1 . Such idioype responses are probably of significance for the antitumor effect of a therapeutic MAb (10).

The rationale for combining MAb17-1A (an anti-colon carcinoma MAb) with GM-CSF in the treatment of CRC is based on the pleiotropic effects of GM-CSF, including mobilization of progenitors from the bone marrow, increasing the number and cytotoxic capacity of granulocytes and monocyte/macrophages, as well as their cytokine production (11–15). The Fc receptor expression on killer cells is enhanced by GM-CSF (14–16). GM-CSF may also augment the induction of an idiotypic network response by activating antigen-presenting cells (17, 18). The induction of an ab_2 and ab_3 response was markedly enhanced in patients receiving MAb17-1A together with GM-CSF, as compared to those receiving MAb17-1A alone (8).

On the basis of our experience with MAb17-1A immunotherapy *in vivo* (19) and our *in vitro* results on GM-CSF together with MAb17-1A (15), a combined MAb17-1A and GM-CSF treatment protocol for patients with metastatic CRC was imple-

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³ The abbreviations used are: CRC, colorectal carcinoma; MAb, monoclonal antibody; ADCC, antibody-dependent cellular cytotoxicity; GM-CSF, granulocyte macrophage colony-stimulating factor; CR, complete remission; PR, partial response; MR, minor response; SD, stable disease; CEA, carcinoembryonic antigen; MNC, mononuclear cell; PBMC, peripheral blood MNC; NK, natural killer; TBS, Tris-buffered saline; C3, complement factor 3.

Table 1 Clinical characteristics and responses of five biopsied patients treated with MAb17-1A in combination with GM-CSF

Patient no.	Age (yr)	Sex	Primary tumor	Stage at diagnosis ^a	Previous therapy	Metastatic site	Response	Survival (months) ^b
1	65	M	Rectum	D	Surgery	Liver, lymph nodes	PD ^c	18.5
2	51	M	Rectum	D	Surgery	Liver	PD	11
3	66	M	Rectum	B3	Surgery	s.c. (abdominal wall)	MR	9
4	69	M	Transverse colon	B3	Surgery	s.c. (abdominal wall)	PD	11
5	53	M	Sigmoid colon	C2	Surgery	Liver, s.c. (abdominal wall)	PD	17.5

^a Modified Astur-Coller staging.^b Survival from start of therapy.^c PD, progressive disease.

mented. The clinical results of this study have been published elsewhere (3), and here we describe intratumoral morphological and immunohistochemical changes observed in biopsies of metastases during therapy.

PATIENTS, MATERIALS, AND METHODS

Patients. Twenty patients were entered in this Phase II trial of combined MAb17-1A and GM-CSF therapy (3). During treatment, consecutive biopsies could be taken from five patients. The clinical characteristics of these five patients are presented in Table 1. All patients had metastatic CRC and a Karnofsky index of >80%. The primary tumors expressed the antigen CO17-1A on >70% of the tumor cells. One patient had received chemotherapy and irradiation 5 months prior to entering the study, and another had had chemotherapy 2 months before. The other patients were, except for primary surgery, untreated.

Treatment Schedule. Human recombinant GM-CSF (250 µg/m²) produced in *Escherichia coli* (Behringwerke AG, Marburg, Germany; specific activity, 5 × 10⁷ units/mg protein) was given s.c. daily for 10 days. On day 3, 400 mg of MAb17-1A (mouse IgG2a; Centocor, Malvern, PA) were infused iv. for 60 min. The treatment cycle was repeated every fourth week, for a total of four cycles (3).

Criteria for Response. To ascertain CR, PR, MR, and SD, the size of a metastatic lesions, as measured on a computed tomography scan, was calculated as a product of two perpendicular diameters. CR was defined as a complete disappearance of all radiological and biochemical evidence of tumor. A PR denotes a >50% decrease in size and a >50% decrease in the serum concentration of CEA, CA19-9, and CA50. A MR was defined as a 25–50% decrease in at least one tumor lesion and/or a >50% decrease in the serum concentration of CEA, CA19-9, and/or CA50 and no increase (>25%) in any lesion. SD was defined as no significant change (<25%) in the size of all measurable lesions and no significant change (<50%) in the serum concentration of CEA, CA19-9, and CA50 for at least 3 months, preceded by a progressive phase. Progressive disease was defined as a >25% increase in size of at least one measurable lesion and >50% increase in the serum concentration of CEA, CA19-9, and/or CA50 (3).

Isolation of PBMCs. On days 1 and 10 of each cycle of treatment, PBMCs (lymphocytes and monocytes) were obtained by centrifugation of heparinized venous blood on a Ficoll/Isopaque gradient (density = 1.077 g/ml; Pharmacia, Uppsala,

Table 2 Antibodies used for immunohistochemical staining of biopsies from patients of metastatic colorectal carcinoma

Antigen	CD	Antibody
T cells		
Pan-T	3	Leu 4 ^a
T suppressor	8	Leu 2a ^a
T helper	4	Leu 3a ^a
IL-2 receptor	25	Anti-Tac ^b
<i>M</i> _r 15,000 granule-associated protein in cytotoxic T cells & NK cells		TIA-1 ^c
NK cells		
Transmembrane form Fc _Y R111A/Fc _Y R111B	16a	Leu 11 ^a
HNK-1	57	Leu 7 ^a
Macrophages	68	KIM6 ^d
Granulocytes		Neutrophil elastase ^e
Mediators		
C3		Antihuman C3 ^f
Mouse IgG		Biotinylated horse antimouse ^g
Tumor cell markers		
CO 17-1A ^h		MAb17-1A ⁱ
GA 73-3 ^h		MAb GA 73-3 ⁱ
Other MAbs		
Cytokeratin		AE1/AE3 ^j
MHC I		HLA ABC (w6/32) ^k
MHC II		HLA DR (DK22) ^e

^a Becton-Dickinson, Mountain View, CA.^b Serotec, Oxford, England.^c TIA-1 was a generous gift from Stuart F. Schlossman, Department of Rheumatology and Immunology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA.^d Behring AG, Marburg, Germany.^e Dakopatts A/S, Copenhagen, Denmark.^f ICM Immunochemicals, Tumba, Sweden.^g Vector Laboratories, Burlingame, CA.^h Different epitopes on the same molecule. GA 73-3 was used in paraffin sections only for testing the eligibility of patients entering the trial.ⁱ MAb17-1A and MAb GA 73-3 were generous gifts from Prof. Kowalewski, Institute of Biotechnology and Advanced Molecular Medicine, Thomas Jefferson University, Philadelphia, PA.^j Boehringer Mannheim, Mannheim, Germany.

Sweden). The PBMCs were washed three times in Hank's Tris Solution before immunostaining (20).

Immuno-Flow Cytometry Analyses. Subpopulations of MNCs were analyzed by indirect immunofluorescence using absorbed FITC-conjugated goat antimouse antibodies (IgG; Becton Dickinson, Mountain View, CA) or rabbit antimouse antibodies (IgG) F(ab')₂ fragments (Dakopatts A/S, Copenha-

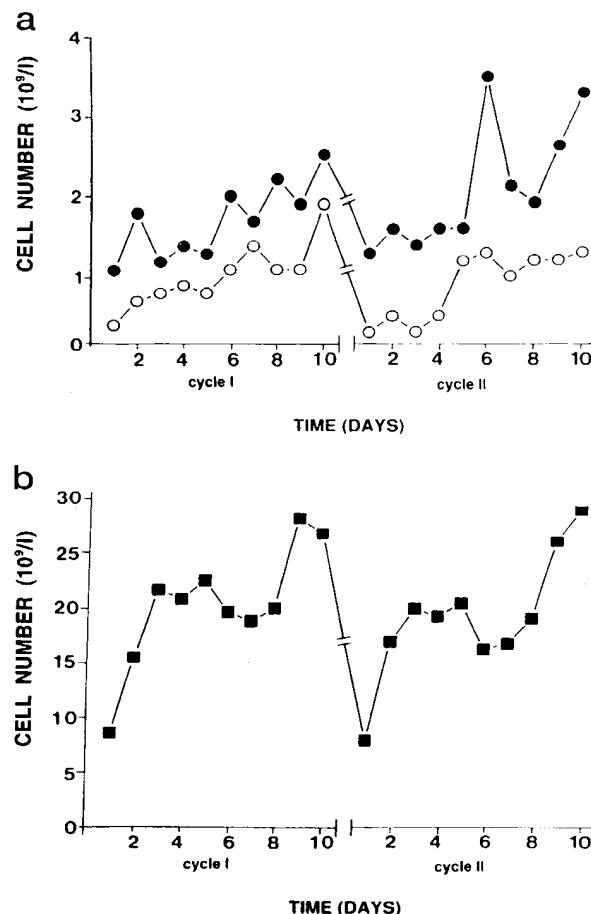


Fig. 1 Total numbers (means) of lymphocytes (a; ●), monocytes (a; ○), and neutrophils (b; ■) in five patients during the first two treatment cycles. GM-CSF was injected for 10 days (days 1–10), and MAb17-1A was injected on day 3.

gen, Denmark). Before staining, the cells were incubated at 37°C for 30 min and washed twice in Hank's Tris Solution, as described previously in detail (21). The following MAbs were used: Leu M3 (CD14), Leu 4 (CD3), Leu 3 (CD4), Leu 2 (CD8), CD25, CD16, and CD57 (Becton Dickinson). Cells (10⁴ per MAb) were analyzed by flow cytometry (FACScan; Becton Dickinson) at 480 nm with a flow rate of <300 cells/s.

Total Blood Cell Counts. At regular intervals, total WBC count was evaluated, as was a differential count of WBCs on May-Grünwald- and Giemsa-stained peripheral blood smears, allowing the calculation of various WBC subtypes from these figures.

Tumor Biopsies. Biopsies of metastatic lesions were taken on days 1 and 10 of treatment cycle I [CID1 (pretreatment biopsy; C = cycle, D = day) and CID10] and cycle II (CIID1 and CIID10), as described previously (22). Biopsies from patient 4 were from hepatic metastases, whereas in the other four patients, metastatic s.c. lesions were obtained. Fresh biopsies

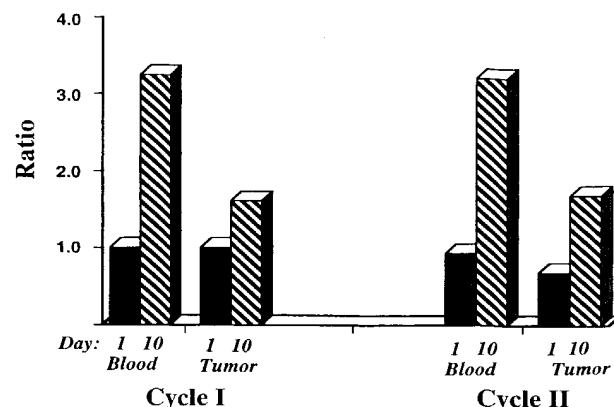


Fig. 2 Columns, relative changes in the ratios (means) of neutrophils in peripheral blood and tumors of five patients during the first two treatment cycles. The values were calculated in comparison to day 1 of cycle I.

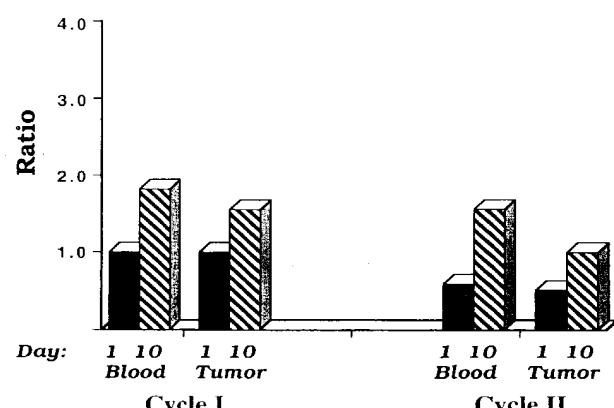


Fig. 3 Columns, relative changes in the ratios (means) of blood CD14⁺ monocytes and tumor CD68⁺ macrophages (CD68 expression tested in tumor) of five patients during the first two treatment cycles. The values were calculated in comparison to day 1 of cycle I.

were snap-frozen in liquid nitrogen and stored at -80°C. Cryostat sections (8 μm) were used for routine H&E staining and immunohistochemistry.

Immunohistochemistry. Immunohistochemistry was carried out on acetone-fixed cryostat sections using a double indirect peroxidase method. The MAbs used for immunohistochemistry are listed in Table 2. Endogenous peroxidase was inhibited by incubation with 500 μg of 30% H₂O₂ in 50 ml of TBS for 30 min, followed by washes in TBS (three 3-min washes). Nonspecific binding was blocked by treatment of sections with normal swine serum (1:10 for 30 min) before incubation with the respective MAb at 4°C overnight. After washing, the sections were incubated with a peroxidase-conjugated rabbit antimouse IgG (1:100; Dakopatts A/S, Copenhagen, Denmark), followed by a peroxidase-conjugated swine antirabbit antibody (1:100), both at room temperature (1 h; Dakopatts,

Table 3 Relative changes in ratios (means) of T lymphocytes and NK cells in the peripheral blood and in the metastases of five colorectal carcinoma patients treated with MAb17-1A and GM-CSF

Ratio ^a	CD3		CD16		CD57	
	PB ^b	Biopsy	PB	Biopsy	PB	Biopsy
CID10/CID1	1.0	2.98	5.53	1.00	1.80	0.70
CIID1/CID1	1.71	1.31	1.23	1.00	1.34	0.75
CIID10/CID1	1.85	2.0	7.68	1.00	1.40	1.00

^a The value at day 1 of cycle I (CID1) was used as baseline (1.0). CID10, cycle I, day 10; CID1, cycle I, day 1; CIID1, cycle II, day 1; CIID10, cycle II, day 10.

^b PB, peripheral blood.

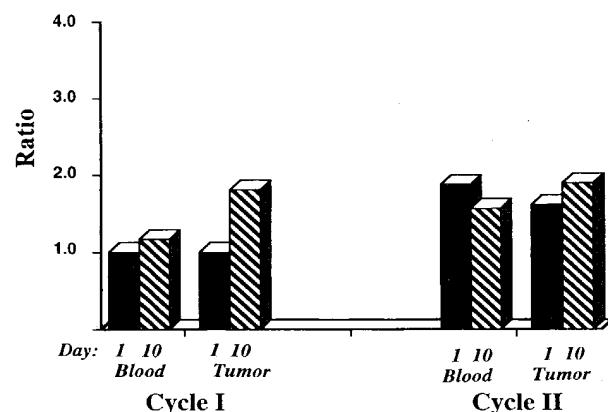


Fig. 4 Columns, relative changes in the ratios (means) of CD8⁺ T cells in peripheral blood and tumor of five patients during the first two treatment cycles. The values were calculated in comparison to day 1 of cycle I.

with a washing (TBS) in between. The conjugates (200 µl) were preabsorbed overnight at 4°C with a solution A (10 µl) of the Immunoglobulin Standard Set (Immunodiagnostic, Boehringer Mannheim, GmbH, Mannheim, Germany) and centrifuged before use. The bound peroxidase was visualized by incubation (5–6 min) with a fresh chromogen substrate solution of diaminobenzidine (30 mg/ml; Sigma Chemical Co., St. Louis, MO) in 50 ml of PBS buffer containing 0.03% H₂O₂. After washing in tap water, the sections were counterstained with Mayer's hematoxylin, dehydrated in increasing grades of alcohol and xylene, and mounted in Eukit (O. Kindler, GmbH & Co., Freiburg, Germany).

Controls. Cryostat sections of a tonsil were immunostained in parallel with the tumor biopsies (positive controls). To evaluate tissue reactivity of the conjugates, TBS was used (TBS control) instead of the primary antibody. TBS control was used for detection of cell-bound mouse MAb17-1A.

Evaluation of Immunohistochemical Staining. Coded biopsies were evaluated without knowledge of the patient's clinical status or the time of the biopsy. A 1-cm grid (graticule) with 100 crosses (+) was inserted into the eye piece ($\times 12.5$) of the microscope, and the relative frequencies of "hits" (+) over tumor cells, stroma, and empty space were recorded. Counting was carried out under an oil immersion lens ($\times 100$). A total of 50 fields were counted. The infiltrating cells positive for par-

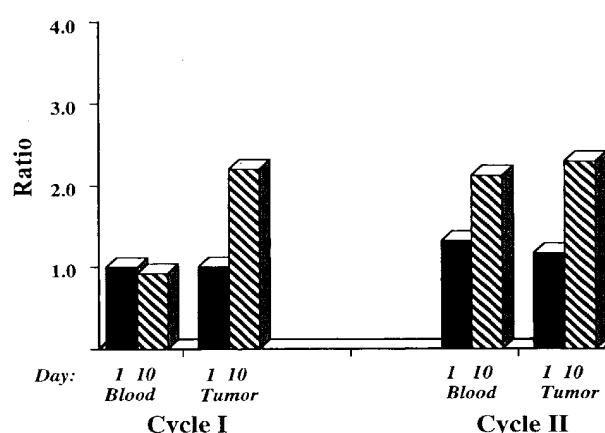


Fig. 5 Columns, relative changes in the ratios (means) of CD4⁺ T cells in peripheral blood and tumors of five patients during the first two treatment cycles. The values were calculated in comparison to day 1 of cycle I.

ticular phenotypes were counted in the stroma of the tumor in that field. The total number of hits on the stroma was divided by 100, which was the total number of stromal fields. The total number of MNCs in 50 fields was counted. The total number of MNCs divided by the total number of stromal fields was the average number of MNCs in one stromal field (unit).

The number of various WBC phenotypes in the pretreatment biopsy (CID1) and blood was considered as a baseline value. Ratios were calculated by comparing the baseline value (CID1) in the biopsy and the peripheral blood to the values in subsequent biopsies and peripheral blood (CID10, CIID1, and CIID10).

Evaluation of Complement Staining. During each treatment cycle, the pretreatment biopsy was compared to the respective posttreatment biopsy. Because the antigen, CO 17-1A, is strongly expressed in the basement membrane of tumor glands, we searched for complement deposition along the basement membrane of the tumor glands (22). The intensity of staining was scored on the following scale: negative, –; very weak, (+); weak, +; moderate, ++; and strong, +++.

RESULTS

Changes in Blood WBC Subsets. GM-CSF treatment induced an increase in the absolute number of monocytes,

lymphocytes, and neutrophils ($P < 0.001$; Fig. 1). The rise in blood neutrophil counts during both treatment cycles was similar (Fig. 2). A 2–3-fold increase in blood PBMCs, mainly monocytes ($CD14^+$), was seen during the two treatment cycles (Fig. 3), whereas during the first treatment cycle, the numbers of $CD3^+$, $CD8^+$, and $CD4^+$ T cells remained essentially unchanged. However, 4 weeks later, at the start of cycle II, the number of T cells [$CD3^+$ (Table 3), $CD8^+$ (Fig. 4), and $CD4^+$ (Fig. 5)] was higher than baseline. During the second treatment cycle, the number of $CD3^+$ and $CD4^+$ (Fig. 5) T cells but not that of $CD8^+$ T cells (Fig. 4) increased further. Cells expressing the interleukin 2 receptor ($CD25$), mainly lymphocytes, were also augmented at the end of each treatment cycle (data not shown), as were $FcyRIII^+$ cells ($CD16$), whereas $CD57^+$ NK cells were not significantly increased (Table 3).

Immunohistochemistry of Metastases. Eighteen biopsies from the five patients obtained during treatment showed viable tumor cells and were evaluated by immunohistochemistry as described below.

Tumor-associated Antigens and MHC Class I/II Antigens. Throughout the course of treatment, the intensity and the number of viable tumor cells reacting with MAAb17-1A did not change measurably. Tumor cells in all of the metastases were positive for MHC class I but were negative for MHC class II antigens and did not show significant changes during therapy. In most biopsies, there was an increase in the number of intratumoral MNCs expressing MHC class II (HLA-DR) molecules in posttreatment biopsies, as compared to pretreatment biopsies (data not shown).

Tumor-infiltrating Neutrophils. An average of 0.87 neutrophil was seen per unit area of stroma in the pretreatment biopsies. A rise in neutrophils following therapy was seen during both cycles (Fig. 2). Infiltrating neutrophils were mainly found in areas of necrosis.

Tumor-infiltrating Macrophages. In the pretreatment biopsies, an average of 1.81 macrophages was seen per unit area of stroma. At day 10 of each treatment cycle (CID10 and CIID10), there was an increase in infiltrating $CD68^+$ MNCs, mostly macrophages, as compared to the pretreatment biopsy (CID1; Fig. 3). The variation in the frequency of infiltrating macrophages followed that of blood monocytes (Fig. 3). In the posttreatment biopsies, cells expressing TIA-1 were usually more in number than $CD8^+$ lymphocytes (Fig. 6, *a* and *b*). Thus, from a quantitative consideration, cells other than $CD8^+$ lymphocytes appeared to express TIA-1. These MNCs were probably macrophages, as few, if any, NK cells were seen in the biopsies (Fig. 6c).

Tumor-infiltrating T Cells. In the pretreatment biopsies, an average of 0.92 $CD3^+$ cell (0.42 $CD4^+$ cell and 0.35 $CD8^+$ cell) was observed per unit area of stroma. $CD3^+$ T cells increased in the metastases during both therapy cycles (Table 3). The rise in intratumoral $CD8^+$ T cells (an average of 0.60 $CD8^+$ cell was seen per unit area of stroma in the posttreatment biopsies) appeared less marked compared to the blood levels (Fig. 4). The most intense infiltration of $CD8^+$ cells was noted in the biopsies of CIID10, as compared to the pretreatment biopsies (CID1), *i.e.*, ~5 weeks after initiation of GM-CSF/MAAb17-1A treatment (Figs. 4 and 7).

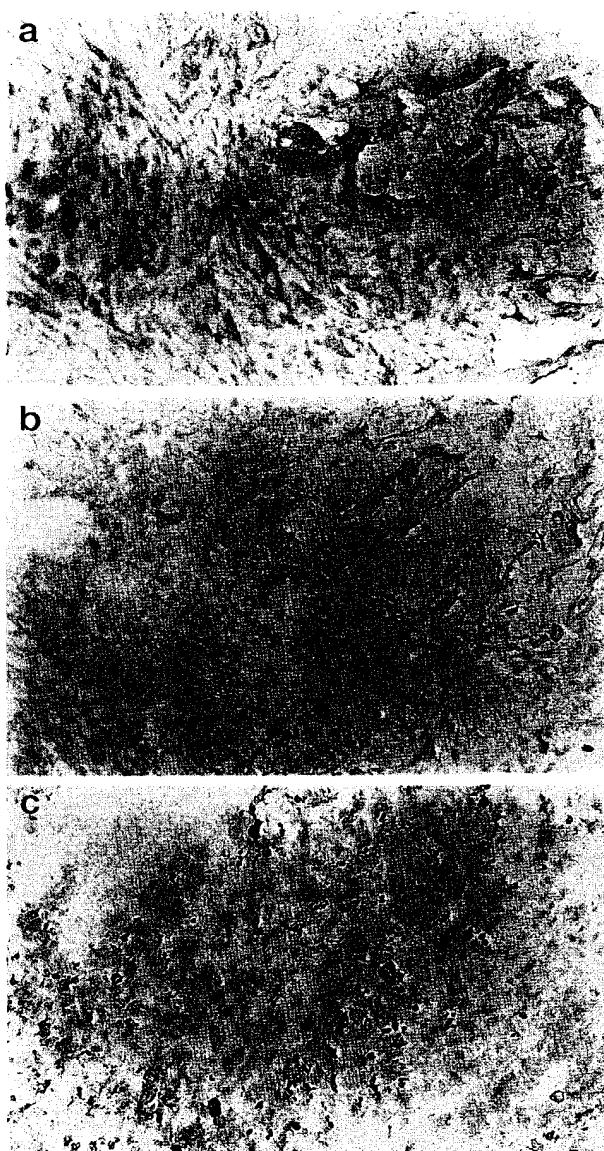


Fig. 6 Consecutive sections of a metastatic CRC lesion immunostained with MAAb TIA-1A (*a*), MAAb CD8 (*b*), and MAAb CD68 (*c*). Note that the number of cells stained by MAAb TIA-1 is more than the number of cells stained by MAAb CD8 but less than that stained by MAAb CD68.

In consecutive sections, $CD8^+$ T cells appeared to stain for TIA-1 (Figs. 6, *a* and *b*). Similar to increase in the number of tumor infiltrating macrophages, neutrophils, and $CD8^+$ cells, there was also an increase in the infiltration of TIA-1 $^+$ cells in the posttreatment biopsy, as compared to the pretreatment biopsy (Fig. 8). These activated $CD8^+$ T cells were mostly located in close proximity to tumor cells. $CD4^+$ T cells were also markedly increased in the tumor during both treatment cycles (an average of 0.92 $CD4^+$ cell was seen per unit area

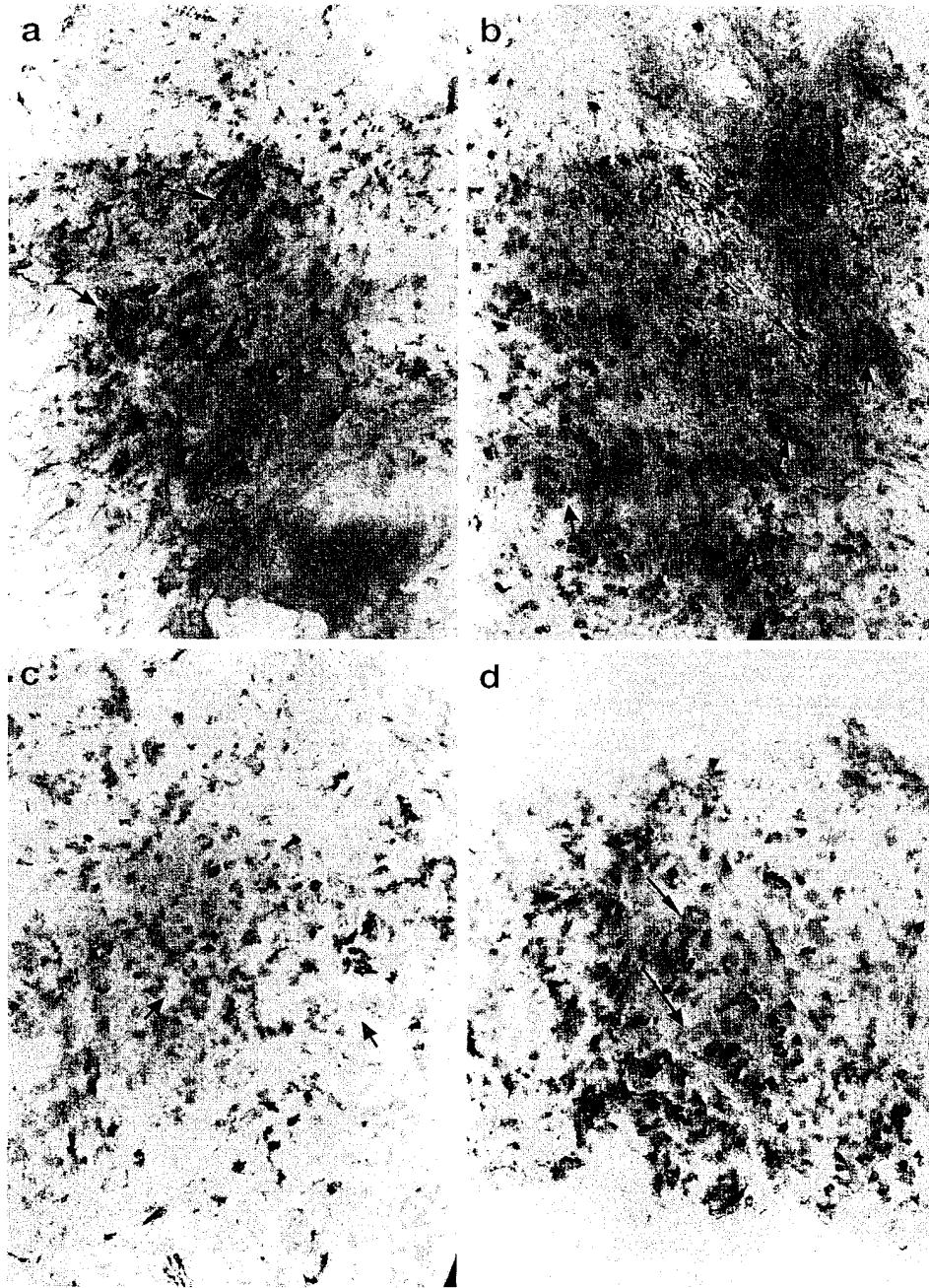


Fig. 7 Sequential biopsies from one patient of a metastatic CRC lesion immunostained with MAb CD8. Note the gradual increase in number of CD8⁺ T cells (dark staining) in the stroma of the tumor during the entire course of treatment. Arrows, lightly hematoxylin-counterstained tumor glands. $\times 250$. *a*, CID1; *b*, CID10; *c*, CIID1; *d*, CIID10.

of stroma in the posttreatment biopsies; Fig. 5) but were in contrast to CD8⁺ cells scattered in the stroma away from the tumor glands. From consecutive sections, it seems as if CD4⁺ T cells were not stained for TIA-1 (data not shown). The intratumoral rise in CD25 (interleukin 2 receptor)-expressing MNCs was not that marked compared to that seen in the blood (data not shown).

Tumor-infiltrating NK Cells. In the pretreatment biopsy, an average of 0.07 NK cell (CD16⁺, CD56⁺, CD57⁺) was seen per unit area of stroma. In contrast to peripheral blood, very few CD16⁺, CD56⁺, and CD57⁺ NK cells were observed to infiltrate the metastases (Table 3) at any time both before and during the entire treatment period. The few infiltrating NK cells present in the metastases were randomly distributed.

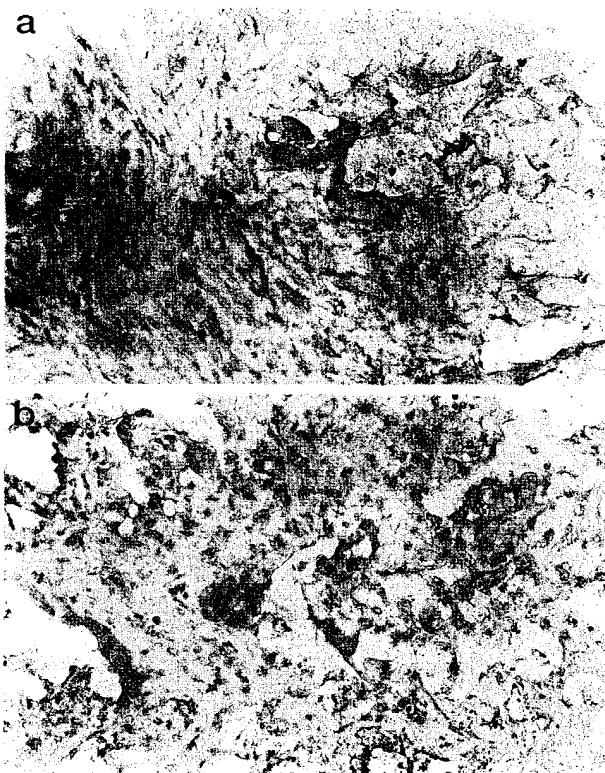


Fig. 8 Immunohistochemical staining of a metastatic CRC lesion stained with MAb TIA-1 demonstrating the increased number of TIA-1⁺ cells in the posttreatment biopsy (*b*) as compared to the pretreatment biopsy (*a*).

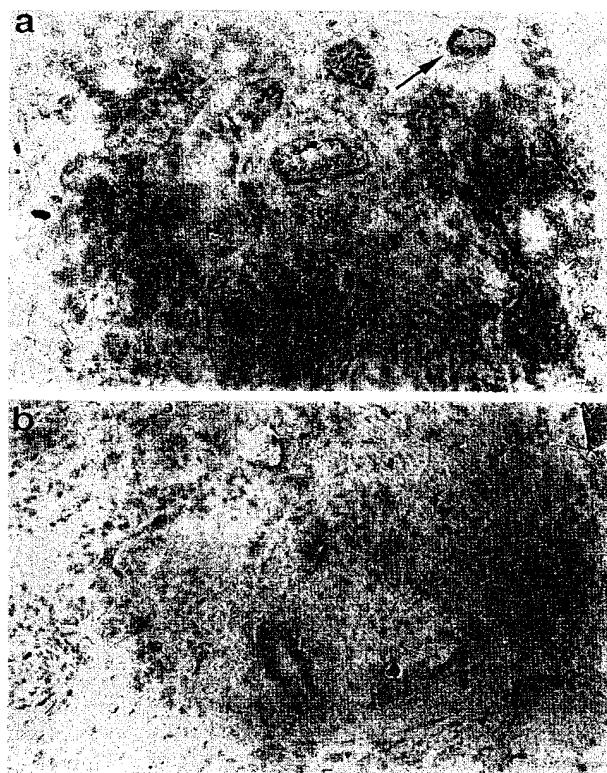


Fig. 9 *a*, consecutive sections from a metastatic CRC lesion of a patient with MR stained with MAb against C3. Moderate deposition of C3 can be seen along the basal region of a tumor gland (arrows) and tumor cells of the adjacent gland seem to be covered by complement. *b*, TBS-stained control section demonstrating the specificity of the C3 staining.

Mouse IgG Antibody (MAb17-1A) and Complement. In all five MAb17-1A-treated patients, mouse IgG antibody was not detected in the day 10 biopsies after treatment cycles I and II (CID10 and CIID10), whereas in four of five patients, post-treatment biopsies (CID10 and CIID10) showed deposition of C3. (Fig. 9) The poorly differentiated metastatic tumor of one patient did not show any complement deposition. The complement deposition was seen mainly along the basement membrane of the tumor glands, where the antigen CO 17-1A was maximally expressed. Occasionally, some complement deposition was also observed in areas of necrosis. In a few areas, tumor cells appeared to be covered by complement. All pretreatment biopsies were negative for C3, except at sites of necrosis. No complement deposition was observed in the biopsy taken prior to the second cycle of treatment (CIID1). The staining intensity of bound complement was mild (+) to moderate (++) after the first treatment (CID10), whereas the intensity was weaker after the second course (CIID10).

DISCUSSION

To our knowledge, this is the first immunohistochemical study of the local immune response in metastases of CRC patients treated with a combination of MAb17-1A and GM-CSF

in relation to changes in neutrophils and MNC phenotypes of peripheral blood.

As reported previously, of the 20 patients treated with a combination of MAb17-1A and GM-CSF, 2 patients (10%) had a CR. The overall response rate (CR + PR + MR + SD) was 30% (2). Of the five patients reported in this study, only one showed a MR; the other four had progressive disease, with a median survival of 11 months. The number of patients biopsied were too few, precluding a statistical clinicopathological correlation. However, the patient that achieved a MR had a moderately differentiated tumor and showed the most pronounced infiltration of CD8⁺ T cells (0.78 cell/unit area of stroma) and CD4⁺ T cells (0.98 cell/unit area of stroma), as well as deposition of C3, in comparison to the other four posttreatment biopsies.

Seven days after a single infusion of 400 mg of MAb17-1A, we were not able to detect MAb17-1A. On the basis of the mild to moderate pattern of complement deposition, mouse IgG should have been bound to the tumors, but the relatively low amount of infused mouse IgG and the delayed interval of the posttreatment biopsy probably precluded its detection by the current staining technique. However, the amount of infused

mouse IgG was obviously sufficient to activate complement. The deposition of C3 at the same site as the maximum expression of CO 17-1A is in accordance with our earlier study using MAb17-1A alone, indicating activation of the classical complement pathway (22). In that study, mouse IgG was consistently detected in biopsies taken 24–72 h after an infusion of 200–400 mg of MAb17-1A. However, in biopsies obtained after infusions of ~2000 mg, mouse IgG could be observed in metastases for up to 3 weeks (22).⁴ In one patient, mouse MAb L6 (IgG2A; antipancarcinoma MAb) could also be identified in tumors up to 19 days after infusions of 400 mg/m² daily for 7 days (>2800 mg/m²; Ref. 24). Thus, the detection of a therapeutic MAb in the tumor seems to be dose dependent. Furthermore, GM-CSF-activated phagocytes may contribute to an enhanced turnover of the MAb by increased phagocytosis. However, mouse IgG was not detected in the macrophages, although it does not exclude a mechanism such as degraded mouse IgG, which may not be detected by this staining technique.

Cells participating in ADCC are monocytes/macrophages, neutrophils, and NK cells. Monocytes/macrophages were increased to a similar degree in the tumor as in the blood following GM-CSF administration. The cells were located around the tumor glands, where MAb17-1A is normally bound (22). In addition, the cells had an activated phenotype capable of lysing tumor cells, as indicated by the presence of cytotoxic granules. Similarly, neutrophils that also can participate in ADCC were seen to infiltrate the tumors of treated patients consequently to their increase in the blood. In contrast, very few NK cells were seen to infiltrate the tumors, although they were clearly increased in the blood. The reason for this selectivity in trafficking of nonspecific killer cells is unclear but should be considered when designing MAb-based therapy protocols in combination with cytokines. Collectively, these data support the notion that ADCC might be a mechanism involved in the destruction of tumor cells by a MAb.

The observed infiltration of T cells, both CD8⁺ and CD4⁺ subsets, into the tumors, particularly CD8⁺ cells at the beginning of the second cycle, seems to indicate a selective stimulatory effect by MAb17-1A and GM-CSF on T cells in between the two treatment cycles. These CD8⁺ cells might represent cytotoxic T cells because they were located around the tumor gland and expressed cytotoxic TIA-1⁺ granules. The increased number of CD4⁺ cells observed in the tumor may reflect an increased helper function. This is in agreement with similar observations reported following MAb therapy in non-Hodgkin's lymphoma, malignant melanoma, and also CRC following MAb17-1A therapy (22, 25, 26). Such an increase in intratumoral CD4⁺ T cells seemed to correlate favorably to the clinical outcome (27, 28). The observed T-cell response in the treated CRC patients could reflect a cellular antitumor response partly induced by the idiotypic network (9, 29–31), in agreement with

observations of an increased humoral idiotypic network response after MAb17-1A and GM-CSF treatment (8).

T cell-mediated tumor cell destruction is dependent on the exposure of target epitopes by MHC class I antigens on the tumor cells, whereas MHC class II molecules (HLA-DR), found on professional antigen presenting cells and also possibly on tumor cells, are required for the activation of helper CD4⁺ T cells. Tumor-infiltrating antigen-presenting cells may process and present tumor antigens from destroyed tumor cells. It is of interest to note that the two patients enrolled in this trial who achieved a CR after treatment (3) had both MHC class I and MHC class II expression on tumor cells,⁵ whereas the five patients in this study expressed MHC class I but not MHC class II on their tumor cells. Nevertheless, the present therapy regimen was effective in recruiting tumor-infiltrating CD4⁺ T cells, a subfraction of which may contain cells that are able to lyse tumor cells (32). Indeed, the lack of class II expression does not preclude induction of a cellular immune response because GM-CSF may activate locally professional antigen-presenting cells that can induce CD4⁺ T cells and, subsequently, initiate a cytotoxic CD8⁺ T-cell response (33).

In summary, combination immunotherapy of GM-CSF and MAb induced a marked recruitment into the tumor metastases of effector cells that are capable of participating in ADCC and CTL activity, further supporting the usefulness of GM-CSF in protocols with therapeutic monoclonal antibodies.

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REFERENCES

- Herlyn, D., Steplewski, Z., Herlyn, M., and Koprowski, H. Inhibition of growth of colorectal carcinoma in nude mice by monoclonal antibody. *Cancer Res.*, **40**: 717–721, 1980.
- Ragnhammar, P., Fagerberg, J., Frödin, J-E., Hjelm, A-L., Lindemalm, C., Magnusson, I., Masucci, G., and Mellstedt, H. Effect of the monoclonal antibody 17-1A and granulocyte monocyte colony stimulating factor in patients with colorectal carcinoma. Long lasting complete remissions can be induced. *Int. J. Cancer*, **53**: 751–758, 1993.
- Ragnhammar, P., Frödin, J-E., Hjelm, A-L., Fagerberg, J., Wersäll, P., Lindemalm, C., Fox, K., Magnusson, I., Ewerth, S., Ahlberg, J., Thor, K., Secher, E., and Mellstedt, H. Different dose regimens of the mouse monoclonal antibody 17-1A alone for therapy of patients with metastatic colorectal carcinoma. *Int. J. Oncol.*, **7**: 1049–1056, 1995.
- Adams, D. O., Hall, T., Steplewski, Z., and Koprowski, H. Tumors undergoing rejection induced by monoclonal antibodies of the IgG2A isotype contain increased numbers of macrophages activated for a distinctive form of antibody-dependent cytotoxicity. *Proc. Natl. Acad. Sci. USA*, **81**: 3506–3510, 1984.
- Herlyn, D. M., and Koprowski, H. Monoclonal anticolon carcinoma antibodies in complement-dependent cytotoxicity. *Int. J. Cancer*, **27**: 769–774, 1981.

⁴ J. Shetye, P. Ragnhammar, M. Lilje fors, B. Christensson, J-E. Frödin, P. Biberfeld, and H. Mellstedt. Metastatic colorectal carcinoma treated with varying doses of MAb17-1A and a combination of MAb17-1A with GM-CSF, manuscript in preparation.

⁵ Unpublished data.

6. Trauth, B. C., Klas, C., Peters, A. M. J., Matzku, S., Möller, P., Falk, W., Debatin, K. M., and Krummer, P. H. Monoclonal antibody mediated tumor regression by induction of apoptosis. *Science* (Washington DC), **245**: 301–305, 1989.
7. Jernic, N. K. Towards a network theory of the immune system. *Ann. Immunol.*, **124**: 373–389, 1974.
8. Ragnhammar, P., Fagerberg, J., Frödin, J-E., Wersäll, P., Hansson, L-O., and Mellstedt, H. Granulocyte monocyte colony stimulating factor augments the induction of antibodies, especially anti-idiotypic antibodies to therapeutic monoclonal antibodies. *Cancer Immunol. Immunother.*, **40**: 367–375, 1995.
9. Fagerberg, J., Frödin, J-E., Wigzell, H., and Mellstedt, H. Induction of an immune network cascade in cancer patients treated with monoclonal antibodies (ab1). I. May induction of ab1-reactive T cells and anti-anti-idiotype antibodies (ab3) lead to tumor regression after mAb therapy? *Cancer Immunol. Immunother.*, **37**: 264–270, 1993.
10. Fagerberg, J., Ragnhammar, P., Lilje fors, M., Hjelm, A-L., Mellstedt, H., and Frödin, J-E. Humoral anti-idiotypic and anti-anti-idiotypic immune responses in cancer patients treated with monoclonal antibody 17-1A. *Cancer Immunol. Immunother.*, **42**: 81–87, 1996.
11. Villeval, J. L., Duhrsen, U., Morstyn, G., and Metcalf, D. Effect of recombinant human granulocyte monocyte colony stimulating factor on progenitor cells in patients with advanced malignancy. *Br. J. Haematol.*, **74**: 36–44, 1990.
12. Munn, D. H., and Cheung, N. K. Phagocytosis of tumor cells by human monocytes cultured in recombinant macrophage colony stimulating factor. *J. Exp. Med.*, **172**: 231–237, 1990.
13. Lieschke, G. J., and Burgess, A. W. Granulocyte colony stimulating factor and granulocyte-macrophage colony stimulating factor. *N. Engl. J. Med.*, **327**: 28–35, 1992.
14. Gasson, J. C., Weisbart, R. H., Kaufman, S. E., Clark, S. C., Hewick, R. M., Wong, G. G., and Golde, D. W. Purified human granulocyte-macrophage colony-stimulating factor: direct action on neutrophils. *Science* (Washington DC), **226**: 1339–1342, 1984.
15. Masucci, G., Wersäll, P., Ragnhammar, P., and Mellstedt, H. Granulocyte-monocyte-colony-stimulating factor augments the cytotoxic capacity of lymphocytes and monocytes in antibody dependent cellular cytotoxicity. *Cancer Immunol. Immunother.*, **29**: 288–292, 1989.
16. Liesveld, J. L., Abboud, C. N., Looney, R. J., Ryan, D. H., and Brennan, J. K. Expression of IgG Fc receptors in myeloid leukaemic cell lines. Effect of colony-stimulating factors and cytokines. *J. Immunol.*, **140**: 1527–1533, 1988.
17. Morrissey, P. J., Bressler, L., Park, L. S., Alpert, A., and Gillis, S. Granulocyte-macrophage colony stimulating factor augments the primary antibody response by enhancing the function of antigen-presenting cells. *J. Immunol.*, **139**: 113–119, 1987.
18. Caux, C., Desutte-Dambuyant, C., Schmitt, D., and Banchereau, J. Granulocyte monocyte colony stimulating factor and tumor necrosis factor cooperate in the generation of dendritic Langerhans cells. *Nature* (Lond.), **360**: 258–261, 1992.
19. Frödin, J-E., Harmenberg, U., Biberfeld, P., Christensson, B., Lefvert, A-K., Riger, Å., Shetye, J., Wahren, B., and Mellstedt, H. Clinical effects of monoclonal antibodies (MAb17-1A) in patients with metastatic colorectal carcinoma. *Hybridoma*, **7**: 309–321, 1988.
20. Böyum, A. A. One stage procedure for isolation of granulocytes and lymphocytes from human blood. *Scand. J. Clin. Lab. Invest. Suppl.*, **97**: 51–76, 1968.
21. Lindemalm, C., Mellstedt, H., Biberfeld, P., Björkholm, M., Christensson, B., Holm, G., Johansson, B., and Sundblad, R. Blood and lymph node T lymphocytes subsets in non-Hodgkin's lymphomas. *Scand. J. Haematol.*, **30**: 68–78, 1983.
22. Shetye, J., Frödin, J-E., Christensson, B., Grant, C., Jacobsson, B., Sundelin, S., Sylvén, M., Biberfeld, P., and Mellstedt, H. Immunohistochemical monitoring of metastatic colorectal carcinoma in patients treated with monoclonal antibodies (MAb17-1A). *Cancer Immunol. Immunother.*, **27**: 154–162, 1988.
23. Anderson, P., Nagler-Anderson, C., O'Brien, C., Levine, H., Watkins, S., Slayter H. S., Blue, M. L., and Schlossman, S. F. A monoclonal antibody reactive with 15 KDa cytoplasmic granule associated protein defines a subpopulation of CD8⁺ lymphocytes. *J. Immunol.*, **144**: 574–582, 1990.
24. Goodman, G. E., Hellström, I., Grodzinsky, L., Nicaise, C., Kulander, B., Hummel, D., and Hellström, K-E. Phase I trial of murine monoclonal antibody L6 in breast, colon and lung cancer. *J. Clin. Oncol.*, **8**: 1083–1092, 1990.
25. Garcia, C. F., Lowder, J., Meeker, T. C., Bendl, J., Levy, R., and Warnke, R. A. Differences in "host infiltrates" among lymphoma patients treated with anti-idiotype antibodies: correlation with treatment response. *J. Immunol.*, **135**: 4252–4260, 1985.
26. Bajorin D. F., Chapman, P. B., Wong, G. Y., Cody, B. V., Cardon-Cardo, C., Dantes, L., Templeton, M. A., Scheinberg, D., Oettgen, H. F., and Houghton, A. N. Treatment with high dose mouse monoclonal (anti GD3) antibody R24 in patients with metastatic melanoma. *Melanoma Res.*, **2**: 355–362, 1992.
27. Cohen, P. J., Lotze, M. T., Roberts, J. R., Rosenberg, S. A., and Jaffe, E. S. The immunopathology of sequential tumor biopsies in patients treated with interleukin-2. Correlation of response with T cell infiltration and HLD-DR expression. *Am. J. Pathol.*, **129**: 208–216, 1987.
28. Rubin, J. T., Elwood, L. J., Rosenberg, S. A., and Lotze, M. T. Immunohistochemical correlates of response to recombinant interleukin-2 based immunotherapy in humans. *Cancer Res.*, **49**: 7086–7092, 1989.
29. Fagerberg, J., Frödin, J-E., Ragnhammar, P., Steinitz, M., Wigzell, H., and Mellstedt, H. Induction of an immune network cascade in cancer patients treated with monoclonal antibodies (ab1). II. Is induction of anti-idiotype reactive T cells (T3) of importance for tumor response to mAb therapy? *Cancer Immunol. Immunother.*, **38**: 149–159, 1994.
30. Fagerberg, J., Hjelm, A-L., Ragnhammar, P., Frödin, J-E., Wigzell, H., and Mellstedt, H. Tumor regression in monoclonal antibody-treated patients correlates with the presence of anti-idiotype-reactive T lymphocytes. *Cancer Res.*, **55**: 1824–1827, 1995.
31. Kosmas, C., Epenetos, A. A., and Courtenay-Luck, N. S. Activation of cellular immunity after intracavitary monoclonal antibody therapy of ovarian cancer. *Cancer* (Phila.), **73**: 3000–3010, 1994.
32. Pham, C. T., and Ley, T. J. The role of granzyme B cluster proteases in cell-mediated cytotoxicity. *Semin. Immunol.*, **9**: 127–133, 1997.
33. Dranoff, G., Jaffee, E., Lazenby, A., Golubek, I., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., and Mulligan, R. C. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA*, **90**: 3539–3543, 1993.